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# Kinetic and Equilibrium Studies of Cyanide Binding by Cytochrome *c* Peroxidase†

James E. Erman

ABSTRACT: The association and dissociation rate constants and the equilibrium dissociation constant for cyanide binding to cytochrome *c* peroxidase have been determined as a function of pH from pH 4 to 10.5 at 25° and 0.15 M ionic strength. The pH dependence of the association rate constant indicates the ionization of hydrocyanic acid and the ionization of a group on the protein, with a p$K_a$ of 5.4, strongly influence the rate of cyanide binding. The association rate constant has a maximum value of $1.1 \times 10^5$ $M^{-1}$ $sec^{-1}$ between pH 6 and 8. Between pH 4 and 8, the dissociation rate constant varies between 0.1 and 1.0 $sec^{-1}$. The pH dependence of the dissociation rate constant in this pH region can be interpreted on the basis of two ionizable groups with p$K_a$ values of 6.5 and 7.3. Above pH 7, the enzyme–cyanide complex undergoes two isomerization reactions, both of which are pH dependent.

The study of fluoride binding to cytochrome *c* peroxidase indicates there is an ionizable group in the native enzyme which strongly influences the binding reaction and perhaps two ionizable groups on the enzyme–fluoride complex which effect the dissociation rate (Erman, 1974). These ionizable groups may play an important role in the mechanism of cytochrome *c* peroxidase catalyzed oxidations. The present study of the reaction between cyanide and cytochrome *c* peroxidase was initiated to confirm the results of the fluoride binding reaction, to determine the influence of the ligand on the apparent p$K_a$ values of the ionizable groups in the complex, and since cyanide forms a much stronger complex at a higher pH than fluoride, to extend the ligand binding studies to higher pH values.

## Experimental Procedure

The experimental procedures were essentially the same as for the fluoride binding studies (Erman, 1974). Cytochrome *c* peroxidase was isolated from baker's yeast and crystallized by dialyzing against distilled water (Yonetani *et al.*, 1966a). Enzyme concentrations ranged from $1 \times 10^{-7}$ to $5 \times 10^{-6}$ M. Reagent grade potassium cyanide was used without further purification. Cyanide solutions were adjusted to the proper pH immediately prior to each experiment with small amounts of $HNO_3$ or KOH and kept stoppered with a minimum of air space above the solution to minimize loss of HCN. Cyanide concentrations ranged from $1 \times 10^{-6}$ to $6.7 \times 10^{-3}$ M. All solutions were 0.15 M ionic strength, adjusted with $KNO_3$, and total buffer concentration was 0.01 M, with acetate, phosphate, and borate buffers used in the appropriate pH region. Stopped-flow studies were carried out in a Durrum-Gibson stopped-flow spectrophotometer thermostatted at 25°. Equilibrium binding studies, slow isomerization kinetics, and spectra were obtained on either a Cary Model 14 or a Cary Model 1501 spectrophotometer with cell compartments thermostatted at 25°.

## Results

*Soret Band of Cytochrome c Peroxidase–Cyanide Complex as a Function of pH.* The absorption spectra of cytochrome *c* peroxidase and its cyanide complex in the Soret region at pH 6 are shown in Figure 1A. The spectrum of the cytochrome *c* peroxidase–cyanide complex has a maximum at 426 nm and an extinction coefficient of 103 $mM^{-1}$ $cm^{-1}$, nearly identical with that previously reported (Yonetani *et al.*, 1966b). The difference spectrum between the cyanide complex and the free enzyme at pH 6 is shown in Figure 1B. The maximum difference occurs at 428 nm and this wavelength was used to monitor the binding reaction in most cases.

Upon addition of unbuffered cytochrome *c* peroxidase to a cyanide solution at pH 8 or higher, there is a rapid formation of the enzyme–cyanide complex, followed by an isomerization reaction, characterized by a shift of the Soret maximum

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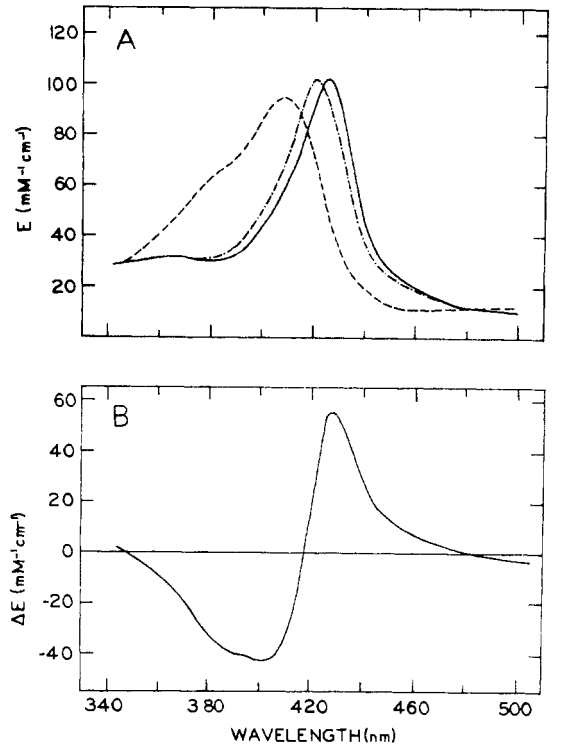



FIGURE 1: (A) Absorption spectra of cytochrome *c* peroxidase at pH 6 (— — — —), acidic cytochrome *c* peroxidase–cyanide complex, pH 6 (————), alkaline cytochrome *c* peroxidase–cyanide complex pH 9 (—●—●—). (B) Difference spectrum between the acidic cytochrome *c* peroxidase–cyanide complex and cytochrome *c* peroxidase at pH 6.

to shorter wavelength. The cyanide complex at pH 9, after the isomerization is complete is also shown in Figure 1A. The Soret maximum is at 421 nm and the maximum extinction coefficient remains the same at 103 $mM^{-1}$ $cm^{-1}$. In this report the two forms of the cyanide complex will be referred to as the acid and alkaline forms. The alkaline cyanide complex is remarkably stable, the absorption spectrum remains essentially unchanged over a period of 24 hr at pH 10. Under the same conditions the native enzyme is irreversibly denatured.

At pH 6.5 and lower, there is no detectable isomerization reaction and only the acidic complex is observed. However the spectrum of the acidic cyanide complex is pH dependent. In Figure 2, the difference spectra between the cyanide complex at pH 6 and other pH values between 4 and 6.5 are shown.

The spectrum of the alkaline cyanide complex is independent of pH from 7.5 to 10. The isomerization rate is very slow at pH 7 and the alkaline cyanide complex was not completely formed after 24 hr. An extrapolation of the change in absorbance to infinite time for the isomerization at pH 7, indicates that the spectrum is the same as at the higher pH values.

*Kinetics of the Initial Enzyme–Cyanide Complex Formation.* Between pH 4 and 6.5, only a single reaction is observed on mixing cytochrome *c* peroxidase and cyanide. For reversible complex formation between the enzyme and cyanide (eq 1),

$$E + CN \underset{k_d}{\overset{k_a}{\rightleftharpoons}} ECN \qquad (1)$$

both the association and dissociation rates must be taken into account. The kinetic studies were carried out under conditions where the cyanide concentration was at least tenfold higher than the enzyme concentration and pseudo-first-order kinetics

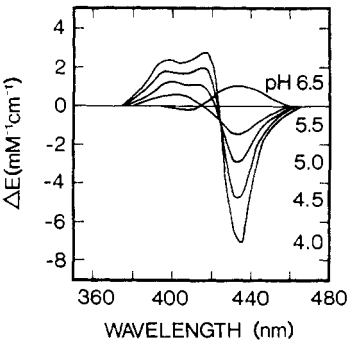



FIGURE 2: Difference spectrum of the acidic cytochrome *c* peroxidase–cyanide complex at the indicated pH values and the complex at pH 6.

were observed. The observed rate constant was dependent on the cyanide concentration according to

$$k_A^{obsd} = k_a[CN]_0 + k_d \qquad (2)$$

where $k_a$ and $k_d$ are defined by eq 1 and $(CN)_0$ is the total, initial cyanide concentration. Plots of $k_A^{obsd}$ *vs.* $(CN)_0$ were linear as predicted by eq 2, the slope giving the value for $k_a$ and the intercept giving the value for $k_d$. At each pH, $k_A^{obsd}$ was determined for at least five cyanide concentrations. The values of $k_a$ and $k_d$ were obtained by fitting $k_A^{obsd}$ to eq 2, using the general-purpose curve-fitting program, KINET, developed at Michigan State University (Dye and Nicely, 1971). Values of $k_a$ and $k_d$, along with estimates of the standard error are given in Table I.

At pH 7 and 7.5, a slow isomerization was detectable, but was so much slower than the binding reactions that it did not interfere with determination of $k_a$ and $k_d$. At pH 7.8 and above, a second, faster isomerization reaction was present, with a rate constant of the same order of magnitude as $k_d$, indicating that, at low cyanide concentrations, the binding reaction and the fast isomerization reaction would be strongly coupled, which in fact was observed. Considering the binding and the fast isomerization, eq 1 can be extended to give

$$E + CN \underset{k_d}{\overset{k_a}{\rightleftharpoons}} ECN_{acid} \underset{k_{-b}}{\overset{k_b}{\rightleftharpoons}} ECN_{int} \qquad (3)$$

where $ECN_{acid}$ represents the acidic enzyme–cyanide complex, the only one observed at low pH, and $ECN_{int}$ represents an intermediate form of the enzyme–cyanide complex. The observation of two isomerization reactions indicates there are at least three forms of the enzyme–cyanide complex. The acidic form is considered to be the initial complex formed and the alkaline enzyme–cyanide complex is considered to be the final form after all isomerizations are complete. The intermediate form represented in eq 3 couples the fast and slow isomerization steps. The mechanism represented by eq 3 is characterized by two reactions which have the rate constants given by eq 4 and 5.

$$k_A^{obsd} = \frac{b + \sqrt{b^2 - 4c}}{2} \qquad (4)$$

$$k_B^{obsd} = \frac{b - \sqrt{b^2 - 4c}}{2} \qquad (5)$$

TABLE I: Rate and Equilibrium Constants for Binding of Cyanide to Cytochrome *c* Peroxidase.[a]

| pH | $k_a$ ($M^{-1}$ $sec^{-1}$) | $k_d$ ($sec^{-1}$) | $K_D = k_d/k_a$ (M) | $K_D$(equil) (M) |
|---|---|---|---|---|
| 4.0 | $(4.4 \pm 0.3) \times 10^3$ | $0.24 \pm 0.02$ | $(5.5 \pm 0.6) \times 10^{-5}$ | $(3 \pm 1) \times 10^{-5}$ |
| 4.2 | $(6.3 \pm 1.6) \times 10^3$ | $0.26 \pm 0.06$ | $(4.1 \pm 1.4) \times 10^{-5}$ | |
| 4.5 | $(1.5 \pm 0.1) \times 10^4$ | $0.17 \pm 0.06$ | $(1.1 \pm 0.4) \times 10^{-5}$ | $(1.5 \pm 0.6) \times 10^{-5}$ |
| 5.0 | $(4.5 \pm 0.4) \times 10^4$ | $0.14 \pm 0.13$ | $(3 \pm 3) \times 10^{-6}$ | $(8 \pm 3) \times 10^{-6}$ |
| 5.5 | $(8.4 \pm 0.3) \times 10^4$ | $0.25 \pm 0.11$ | $(3 \pm 1) \times 10^{-6}$ | $(6 \pm 2) \times 10^{-6}$ |
| 6.0 | $(1.1 \pm 0.1) \times 10^5$ | $0.39 \pm 0.05$ | $(3.6 \pm 0.6) \times 10^{-6}$ | $(6 \pm 3) \times 10^{-6}$ |
| 6.5 | $(1.2 \pm 0.1) \times 10^5$ | $0.51 \pm 0.01$ | $(4.3 \pm 0.1) \times 10^{-6}$ | $(2 \pm 0.6) \times 10^{-6}$ |
| 7.0 | $(1.1 \pm 0.1) \times 10^5$ | $0.90 \pm 0.10$ | $(8 \pm 1) \times 10^{-6}$ | $(2 \pm 0.6) \times 10^{-6}$ |
| 7.5 | $(1.1 \pm 0.1) \times 10^5$ | $0.46 \pm 0.35$ | $(4 \pm 1) \times 10^{-6}$ | $(9 \pm 3) \times 10^{-7}$ |
| 7.8 | $(1.3 \pm 0.1) \times 10^5$ | $0.00 \pm 0.41$ | | |
| 7.9 | $(9.7 \pm 1.0) \times 10^4$ | $0.09 \pm 0.11$ | $(1 \pm 2) \times 10^{-6}$ | |
| 8.5 | $(8.7 \pm 0.4) \times 10^4$ | $0.10 \pm 0.31$ | $(1 \pm 4) \times 10^{-6}$ | |
| 9.0 | $(1.2 \pm 0.1) \times 10^5$ | $1.1 \pm 0.8$ | $(9 \pm 7) \times 10^{-6}$ | |
| 9.5 | $(5.2 \pm 0.5) \times 10^4$ | $1.1 \pm 0.2$ | $(2.1 \pm 0.4) \times 10^{-5}$ | |
| 9.8 | $(1.6 \pm 0.3) \times 10^4$ | $3.7 \pm 0.3$ | $(2.3 \pm 0.5) \times 10^{-4}$ | |
| 10.4 | $(3.2 \pm 0.7) \times 10^3$ | $9.7 \pm 0.7$ | $(3.0 \pm 0.7) \times 10^{-3}$ | |

[a] 25°, 0.15 M ionic strength.

where

$$b = k_a[CN] + k_d + k_b + k_{-b} \quad (6)$$

and

$$c = k_d k_b + k_a[CN](k_b + k_{-b}) \quad (7)$$

In the limit of high cyanide concentration, it can be shown that eq 4 and 5 simplify to eq 2 and 8 (Hammes and Schimmel, 1970).

$$k_B^{obsd} = k_b + k_{-b} \quad (8)$$

Using sufficiently high cyanide concentrations makes the binding reaction much faster than the isomerization reaction and effectively uncouples the two reactions. Again a plot of $k_A^{obsd}$ *vs.* [CN] at high cyanide concentrations gives values of $k_a$ and $k_d$. Unfortunately, the cyanide concentration cannot be decreased sufficiently low to obtain a precise value of $k_d$ at pH values between pH 7.8 and 9 due to the coupling with the isomerization reaction, resulting in the large error for the $k_d$ values in this pH region. At pH values greater than 9, $k_d$ is larger than the isomerization rate and better extrapolations are obtained. Between pH 9.5 and 10.5, reasonably accurate $k_d$ values are obtained.

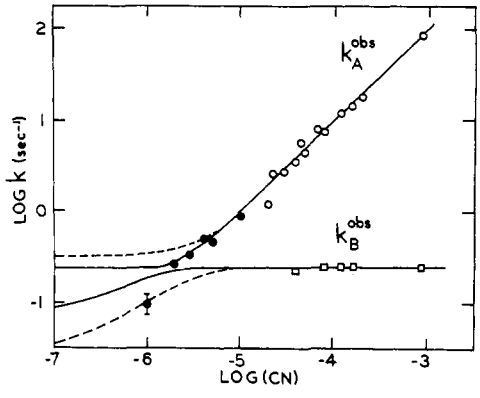



FIGURE 3: Log $k_A^{obsd}$ and log $k_B^{obsd}$ plotted as a function of log [CN]. At low cyanide concentration only a single reaction was observed indicated by the solid circles. At high cyanide concentrations, two reactions were observed, indicated by the open circles and open squares. The solid lines are calculated from eq 4 of the text with $k_a = 9.7 \times 10^4$ $M^{-1}$ $sec^{-1}$, $k_d = 0.09$ $sec^{-1}$, $k_b = 0.00$ $sec^{-1}$, and $k_{-b} = 0.25$ $sec^{-1}$. The dashed lines were calculated from eq 5 with the same values of $k_a$ and $k_d$ but with $k_b = 0.15$ $sec^{-1}$ and $k_{-b} = 0.10$ $sec^{-1}$.

Figure 3 is a log–log plot of the observed rate constants as a function of cyanide concentration from $10^{-6}$ to $10^{-3}$ M at pH 7.9. At the lowest concentrations only a single reaction could be detected by the stopped-flow technique. The values for the rate constant when only a single reaction was observable are indicated by the solid circles in Figure 3. At $2 \times 10^{-5}$ M cyanide, the second reaction was detectable but the amplitude was too small to determine the rate constant. Above $4 \times 10^{-5}$ M cyanide, the rate constants for both reactions could be determined. The concentration-dependent rate is indicated by the open circles and the concentration-independent rate is indicated by the open squares in Figure 3.

*Dependence of $k_a$ and $k_d$ on pH.* The pH dependence of $k_a$ and $k_d$ is shown in Figure 4. The association rate constant is

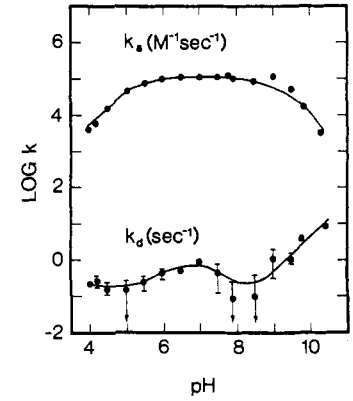



FIGURE 4: Plot of log $k_a$ (upper curve) and log $k_d$ (lower curve) as a function of pH. Standard error bars are indicated when the standard error exceeds the size of the symbols. The solid lines are calculated according to a mechanism presented in the text.

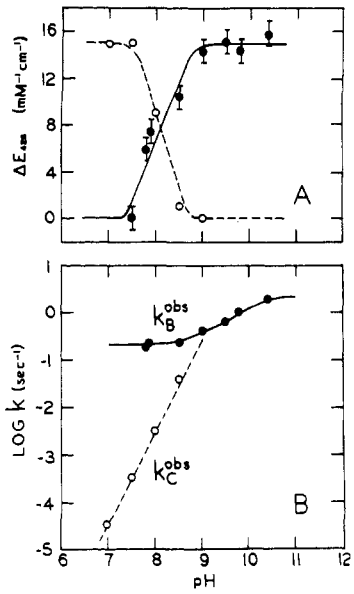



FIGURE 5: (A) Amplitude of the two isomerization reactions as a function of pH. The solid circles give the amplitude for the $k_B^{obsd}$ reaction determined from stopped-flow measurements. The open circles give the amplitude of the $k_c^{obsd}$ reaction from spectrophotometric determinations. (B) Values of $k_B^{obsd}$ (solid circles) and $k_c^{obsd}$ (open circles) as a function of pH.

independent of pH between pH 6 and 8.5. At the pH extremes, the association rate constant decreases. The pH dependence of $k_d$ is more complex, going through a maximum at pH 7, through minima near pH 4.5 and 8.5, then increasing again at the pH extremes.

*Isomerization of the Enzyme–Cyanide Complex.* During the course of the spectral and kinetic studies on the formation of the enzyme–cyanide complex, two isomerization reactions were detected. One, a fast isomerization observed in the stopped-flow kinetic studies is only seen above pH 7.8. The value for this isomerization rate was determined at high cyanide concentrations according to eq 8. Values of $k_B^{obsd}$ are given in Table II. Both the value of $k_B^{obsd}$ and the amplitude

TABLE II: Cytochrome *c* Peroxidase–Cyanide Complex Isomerization Rate Constants.[a]

| pH | $k_B^{obsd}$ (sec$^{-1}$) | $k_C^{obsd}$ (sec$^{-1}$) |
|---|---|---|
| 7.0 | | $3.5 \times 10^{-5}$ |
| 7.5 | | $3.3 \times 10^{-4}$ |
| 7.8 | 0.18 ± 0.02 | |
| 7.9 | 0.25 ± 0.05 | |
| 8.0 | | $3.2 \times 10^{-3}$ |
| 8.4 | 0.24 ± 0.02 | |
| 8.5 | | $4.0 \times 10^{-2}$ |
| 8.9 | 0.43 ± 0.07 | |
| 9.4 | 0.61 ± 0.06 | |
| 9.8 | 1.00 ± 0.04 | |
| 10.4 | 1.94 ± 0.20 | |

[a] 25°, 0.15 M ionic strength.

of the reaction are pH dependent and these quantities are plotted in Figure 5. The solid points in Figure 5A give the amplitude of the reaction in terms of an extinction coefficient.

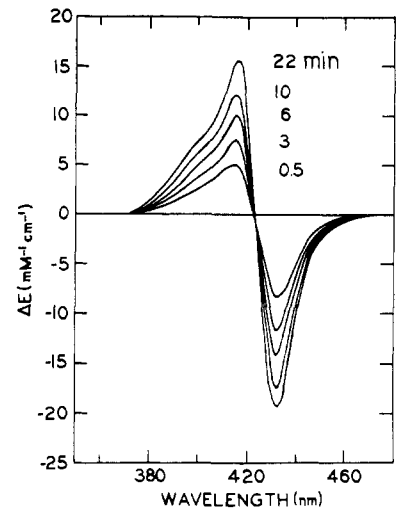



FIGURE 6: Difference spectrum of the reaction mixture upon addition of cytochrome *c* peroxidase to a 0.1 M cyanide solution, pH 8, ionic strength 0.15 M, and the acidic cytochrome *c* peroxidase–cyanide complex at pH 6 as a function of time.

The maximum amplitude occurs between pH 9 and 10.5 but decreases to zero at pH 7.5. The value of $k_B^{obsd}$ as a function of pH is shown by the solid points in Figure 5B. In general $k_B^{obsd}$ increases with pH.

The second isomerization is much slower and was detected by the time dependence of the enzyme–cyanide complex spectrum. Figure 6 shows the time course of the reaction at pH 8 on addition of an unbuffered cytochrome *c* peroxidase solution, near pH 5.5, to a buffered cyanide solution. The spectral changes are given as difference spectra between the reaction mixture and the acidic enzyme–cyanide complex at pH 6; 0.5 min after mixing, the fast isomerization has been completed, giving the initial difference spectrum. This is followed by the second isomerization which is complete in about 20 min at this pH. The difference spectrum shown in Figure 6 at 22 min represents the difference spectrum between the alkaline enzyme–cyanide complex at pH 8 and the acidic enzyme–cyanide complex at pH 6. This difference spectrum is independent of the pH of the alkaline enzyme–cyanide complex.

Semilogarithmic plots of the change in absorbance as a function of time allows evaluation of the observed first-order rate constant for this isomerization, $k_C^{obsd}$, which is tabulated in Table II at various pH values. The rate constant, $k_C^{obsd}$, and the amplitude of this second isomerization are pH dependent and are represented by the open circles in Figure 5. The maximum amplitude of this reaction is between pH 7 and 7.5, then decreases to zero above pH 9. The value of $k_C^{obsd}$ decreases with the square of the hydrogen ion concentration as seen in Figure 5B.

*Equilibrium Dissociation Constants for the Cytochrome c Peroxidase Cyanide Complex.* The equilibrium dissociation constant for the reaction in eq 1 is given by

$$K_D = (\bar{E})(\bar{CN})/(\bar{ECN}) \quad (9)$$

where $(\bar{E})$, $(\bar{CN})$, and $(\bar{ECN})$ represent the equilibrium concentrations, in all forms, of free enzyme, free cyanide, and the enzyme–cyanide complex, respectively. The values of $K_D$ as a function of pH were determined spectrophotometrically. In terms of changes in absorbance

$$(\bar{E})/(ECN) = (\Delta A_\infty - \Delta A)/\Delta A \quad (10)$$

where $\Delta A_\infty$ is the change in absorbance between complete complex formation and free enzyme and $\Delta A$ is the change in absorbance at intermediate values of complex formation and free enzyme. Knowing the total enzyme concentration, the total cyanide concentration, the ratio given by in eq 10, and assuming a one to one complex between enzyme and cyanide, the equilibrium concentration of all species can be determined. $K_D$ was evaluated from the slopes of Scatchard plots (Scatchard, 1949). A typical plot is shown in Figure 7. Values of $K_D$ are given in Table I. A plot of $K_D$ as a function of pH is shown in Figure 8 along with values of $K_D$ calculated from $k_a$ and $k_d$ determined from the kinetics of cyanide binding. The agreement is good below pH 6, with deviations occurring between pH 6.5 and 7.5. Above pH 7.5, the isomerization reaction and the instability of the free enzyme precluded determination of the equilibrium dissociation constants although a value could still be calculated from the kinetic data. The calculated values of $K_D$ above pH 7 do not include the isomerization reactions.

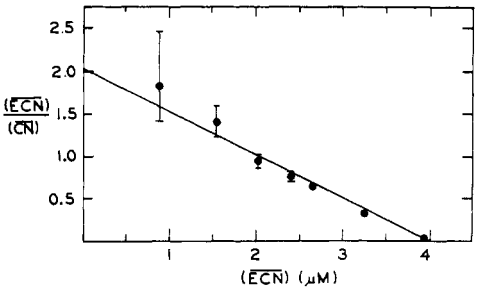



FIGURE 7: Scatchard plot for the binding of cyanide to cytochrome *c* peroxidase at pH 6.5.

## Discussion

The pH dependence of $k_a$ (Figure 4) can be explained on the basis of a single ionization on cytochrome *c* peroxidase with a $pK_a$ of 5.4 and the ionization of hydrocyanic and which has a $pK_a$ of 9.0 at 0.15 M ionic strength, 25° (Izatt *et al.*, 1962). The question of whether the protonated or unprotonated form of the ligand binds has been discussed in detail in the report on fluoride binding to cytochrome *c* peroxidase (Erman, 1974). In that report, the favored interpretation is that the protonated form of the ligand reacts with the enzyme.

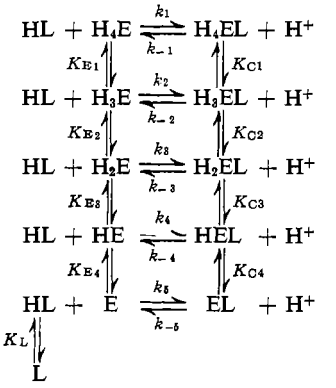
Just as with the fluoride complex, the variation of the cyanide dissociation rate constant with pH is rather small between pH 4 and 9. It is difficult to determine whether this pH variation is due to the influence of the general state of ionization and conformation of the protein or to ionization of specific groups near the heme site. However since it is not possible to quantitatively determine the effect of net charge and conformation on $k_d$, all of the pH variation is attributed to specific ionizations. The simplest mechanism to account for the pH variation of $k_a$ and $k_d$ and satisfy microscopic reversibility is shown in Scheme I and eq 11 and 12. The best-fit parameters

$$k_a = \frac{k_1 + k_2\frac{K_{E1}}{[H^+]} + k_3\frac{K_{E1}K_{E2}}{[H^+]^2} + k_4\frac{K_{E1}K_{E2}K_{E3}}{[H^+]^3} + k_5\frac{K_{E1}K_{E2}K_{E3}K_{E4}}{[H^+]^4}}{\left(1 + \frac{K_L}{[H^+]}\right)\left(1 + \frac{K_{E1}}{[H^+]} + \frac{K_{E1}K_{E2}}{[H^+]^2} + \frac{K_{E1}K_{E2}K_{E3}}{[H^+]^3} + \frac{K_{E1}K_{E2}K_{E3}K_{E4}}{[H^+]^4}\right)} \quad (11)$$

$$k_d = \frac{[H^+]\left(k_{-1} + k_{-2}\frac{K_{C1}}{[H^+]} + k_{-3}\frac{K_{C1}K_{C2}}{[H^+]^2} + k_{-4}\frac{K_{C1}K_{C2}K_{C3}}{[H^+]^3} + k_{-5}\frac{K_{C1}K_{C2}K_{C3}K_{C4}}{[H^+]^4}\right)}{1 + \frac{K_{C1}}{[H^+]} + \frac{K_{C1}K_{C2}}{[H^+]^2} + \frac{K_{C1}K_{C2}K_{C3}}{[H^+]^3} + \frac{K_{C1}K_{C2}K_{C3}K_{C4}}{[H^+]^4}} \quad (12)$$

are given in Table III. Only the equilibrium dissociation constants for the two groups which ionize within the pH range studied, $K_{E1}$, $K_{E2}$, $K_{C1}$, and $K_{C2}$ in the enzyme and in the complex respectively are specified by fitting the pH dependence of $k_a$ and $k_d$. The two additional groups, $K_{C3}$ and $K_{C4}$ in the complex, are required to quantitatively fit the dissociation rate data. It seems more likely that these apparent ionizations represent a complex process involving substantial changes in the conformation of the enzyme cyanide complex in the high pH region. Values of $K_{C3}$ $K_{C4}$, and $k_{-5}$ can have a range of interdependent values. The values used to calculate the solid lines in Figures 4 and 8 are given in parentheses in Table III.

SCHEME I




Several mechanisms can be postulated to account for the two isomerizations of the cytochrome *c* peroxidase–cyanide complex but the most straight forward is an extension of eq 3 to include a second isomerization in sequence.

$$E + CN \underset{k_d}{\overset{k_a}{\rightleftharpoons}} ECN_{acid} \underset{k_{-b}}{\overset{k_b}{\rightleftharpoons}} ECN_{int} \underset{k_{-c}}{\overset{k_c}{\rightleftharpoons}} ECN_{alk} \quad (13)$$

The initial enzyme–cyanide complex formed is equated with the acid form, the only one observed below pH 6.5, followed by

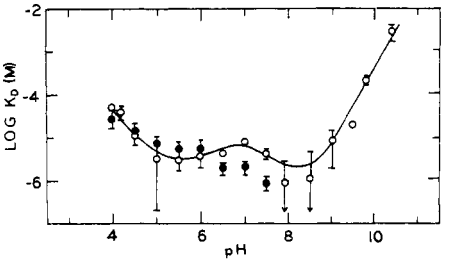



FIGURE 8: Plot of log $K_D$ as a function of pH for the dissociation of the acidic cytochrome *c* peroxidase–cyanide complex to form cytochrome *c* peroxidase and cyanide. The solid circles are values of $K_D$ determined from equilibrium measurements, the open circles are values of $K_D$ calculated from the values of $k_a$ and $k_d$ determined in the stopped-flow studies and the line was calculated from a mechanism presented in the text.

TABLE III: Rate and Equilibrium Constants for the Mechanism in the Text Used to Fit the pH Dependence of $k_a$ and $k_d$.[a]

| | | | |
|---|---|---|---|
| $k_1$ | $1.8 \times 10^3$ | $k_{-1}$ | $8 \times 10^2$ |
| $k_2$ | $1.1 \times 10^5$ | $k_{-2}$ | $5 \times 10^5$ |
| $k_3$ | $1.1 \times 10^5$ | $k_{-3}$ | $2.6 \times 10^7$ |
| | | $k_{-5}$ | $(4.4 \times 10^{14})$ |
| $K_{E1}$ | $3.2 \times 10^{-6}$ | | |
| $K_{E2}$ | $3.2 \times 10^{-6}$ | $K_{C2}$ | $5.0 \times 10^{-8}$ |
| $K_{C1}$ | $3.2 \times 10^{-7}$ | $K_{C3}$ | $(1 \times 10^{-12})$ |
| | | $K_{C4}$ | $(1 \times 10^{-12})$ |

[a] $k$ values are in $M^{-1}$ $sec^{-1}$. $K$ values are in M. The parameters $k_4$, $k_5$, $k_{-4}$, $K_{E3}$, and $K_{E4}$ did not influence the observed rate constants.

the rapid isomerization, characterized by $k_B^{obsd}$, to an intermediate form of the complex, followed by the second isomerization, characterized by $k_C^{obsd}$, to the alkaline form of the enzyme–cyanide complex. Equating the first isomerization step with $k_B^{obsd}$ followed by $k_C^{obsd}$ is consistent with the fact that both isomerization reactions are observed between pH 7.8 and 8.5. At pH 9 and higher, only one isomerization reaction is detected. If $k_C^{obsd}$ is assumed to follow the same pH dependence above pH 8.5 as it follows below pH 8.5, $k_C^{obsd}$ will become larger than $k_B^{obsd}$ at pH values greater than 9. The second isomerization is faster than the first and $ECN_{acid}$ is converted to $ECN_{alk}$ with only a vanishingly small amount of $ECN_{int}$ present. As a consequence the fast step, $k_C^{obsd}$, is not detectable by the stopped-flow method.

The equilibrium between $ECN_{acid}$ and $ECN_{int}$ favors the acid form at low pH but as the pH is raised, the relative concentration of $ECN_{int}$ increases and is converted to $ECN_{alk}$ by the second isomerization. The second isomerization strongly favors the alkaline form of the enzyme–cyanide complex and at equilibrium, $ECN_{alk}$ is the only form present in detectable quantities above pH 7.5.

The study of cyanide binding to cytochrome *c* peroxidase has shown both similarities and differences with respect to fluoride binding. The same ionizable groups on the enzyme influence both the association and dissociation rate constants. The $pK_a$ values of these groups are perturbed to different extents by the two ligands. The spectrum of the cyanide complex and the native enzyme are both sensitive to the state of protonation between pH 4.5 and 7.5, while the fluoride complex is not. The cyanide complex undergoes two isomerization reactions at alkaline pH, which are not observed in the fluoride complex, possibly due to the weak binding of fluoride in the alkaline pH region. In fact, the isomerization has not been reported in the cyanide complexes of other heme proteins (Dolman *et al.*, 1968; Ellis and Dunford, 1968; Ver Ploeg *et al.*, 1971) and is apparently due changes in the protein structure of cytochrome *c* peroxidase. The stability of the alkaline enzyme–cyanide complex should be useful in studying the physical properties of the enzyme above pH 8, a region not accessible with the native enzyme due to irreversible denaturation.

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